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EUROPEAN PATENT APPLICATION

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(54) Improved control of expression of heterologous genes from lac operated promoters.

(57) The invention is a process for controlling the expression of heterologous genes from lac -operated promoters by removing the CAP binding site and lac promoter from the lac operon. Illustrated is the fusion of the lacZ , Y , and A genes of the lac operon to the 3' end of the lacI structural gene. This elimination of the natural regulatory elements of the lac operon results, advantageously, in the production of lac operon gene products in a constitutive mode from the lacI promoter.

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IMPROVED CONTROL OF EXPRESSION OF HETEROLOGOUS GENES FROM LAC OPERATED PROMOTERS

Background of the Invention

5   The lactose (lac) operon consists of three protein products under the control of a lac promoter-operator. These gene products are β-galactosidase (Z) permease (Y), and thiogalactoside transacetylase (A). The protein product of the lacI transcript (repressor), an independent gene product, interacts with the operator of the lac operon and keeps synthesis off until allolactose (1,6-0-β-D-galactopyranosyl-D-glucose), a product of the β-galatosidase reaction, accumulates in the cell and binds to the repressor. The allolactose repressor complex has a changed conformation, allowing the repressor to be displaced from the operator.

10   RNA transcription then begins from the lac promoter (Beckwith, J. [1987] In *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology . Vol. 2, Neidhardt, F.C., Editor in Chief, American Society for Microbiology, Washington, D.C.).

   A few molecules of the lac operon transcript are present in E. coli , even in the absence of lactose. Hence, permease and β-galactosidase are always present, at least at a low level. Allolactose, the natural

15   inducer of the operon, is made in the cell when lactose (1,4-0-β-D-galactopyanosyl-D-glucose) enters the cell by the permease reaction and is converted through transgalactosidation by β-galactosidase into allolactose (Freifelder, D., [1987] Molecular Biology . Jones and Bartlett Publishers, Inc., Portola Valley, CA; Beckwith, supra). It was calculated that greater than 20% of the lactose acted upon by β-galactosidase is converted to allolactose (Jobe, A., Bourgeois, S., [1972] J. Mol. Biol. 69 :397-408). The majority of the

20   remaining lactose is converted to glucose and galactose. Allolactose is a better substrate for β-galactosidase than lactose (Jobe and Bourgeois, supra , and is itself rapidly converted to glucose and galactose.

   Another control element of the lac operon is catabolite repression. In the presence of glucose the cell is able to repress many operons. For example the lac operon is only transcribed at 2% of its maximum level in the presence of glucose (Beckwith, supra).

25   When several β-galactosides were compared for their ability to induce the lac operon in vivo , lactose was found to act as a very poor effector molecule. The synthetic non-metabolized β-galactoside, isopropyl-β-D-thiogalactoside (IPTG), was found to induce the lactose operon 5 times better than lactose (Monod, J., G. Cohen-Bazire and M. Cohn [1951] Biochim. Biophys. Acta. 7 :585-599). Yet, allolactose itself is as good an inducer of the operon as IPTG (Muller-Hill, B., H.V. Rickenberg and K Wallenfels [1964] J. Mol. Biol. 10

30   :303-318; Jobe and Bourgeois, supra).

   Given the efficiency of lactose conversion to allolactose, one might expect lactose to work very well as an inducer of the lac operon. However, prior to the present disclosure, this has never occurred in practice (Monod et al., supra).

   The current way to control the expression of heterologous genes from the lac promoter or lac

35   concensus promoters such as tac (Rezinkoff, W.S. and W.R. McClure [1986] Maximizing Gene Expression , W. Reznikoff and L. Gold, eds., Butterworth Publishers, Stoneham, MA) is to have enough lac repressor present in the cell, so that transcription from the tac promotor is off until IPTG or another proper inducing β-galactoside is added to the cell. Although IPTG is the current inducer of choice, it is expensive and has been labeled a potential carcinogen. Thus, there is a need to replace IPTG in commercial systems where

40   control of the expression of heterologous genes from lac operated promoters is used.

Brief Summary of the Invention

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   The subject invention concerns an improved method for controlling the expression of heterologous genes from lac operated promoters. Specifically, the subject invention concerns a method for controlling the expression of heterologous genes from lac operated promoters which comprises removing the CAP binding site and lac promoter/operator from the lac operon. The subject invention is exemplified herein by use of a

50   novel recombinant DNA construct comprising the lacZ , Y and A genes of the lac operon, to control the expression of heterologous genes from lac operated promoters. The lacZ , Y , and A genes of the lac operon were fused to the 3' end of the lacI structural gene, thereby eliminating all of the natural regulatory elements of the lac operon. By doing this, lac operon gene products are produced in a constitutive mode from the lacI promoter and are not responsive to catabolite repression or to allolactose induction. When lactose is added, the constitutively synthesized β-galactosidase converts as much as 20% to allolactose,

which in turn derepresses the tac promoter and any associated heterologous gene. As long as the allolactose concentration in the cell is above 10^{-5} M the tac promoter provides high expression of the protein of interest. The complete DNA sequence of the transcriptional fusion of the lacI gene to the lacZ , Y , and A genes of the subject invention is as shown in Table 1.

The novel lacIZYA operon (Table 1) can be inserted in any plasmid, or it can be inserted into any microbial chromosome to improve control of heterologous gene expression from a lac operated promoter (as long as a promoter is present to drive lacIZYA transcription). It will be apparent to a person skilled in the art that other constructs can be used to fuse the genes.

The lac operator, which has very high affinity for the lacI repressor (K_{DNA} = 2-5x10^{13} M^{-1}), is a sequence 21 b.p. long as follows; AATTGTGAGCGGATAACAATT (Barkley, M.D. and S. Bourgeois [1978] In The Operon Miller, J.H. and W.S. Reznikoff, eds. Cold Spring Harbor). Many mutations within the lac operator exist which have higher or lower affinities to the lacI repressor (Barkley and Bourgeois, supra ; Sadler, J.R. et al. [1983] Proc. Natl. Acad. Sci. USA 80 :6785-6789; Simons, A. et al. [1984] Proc. Natl. Acad. Sci. USA 81 :1624-1628). Many mutations exist within the lacI repressor that allow for greater affinity to the U operator with no significant effect on derepression (Barkley and Bourgeois, supra). Using any of the many different combinations of lac operator and lacI repressor, the control of heterologous gene expression is apparent to a person skilled in the art. Many different promoters (eukaryotic as well as prokaryotic) have been placed under lac operator regulation (Yansura, D.G. and D.J. Henner [1984] Proc. Natl. Acad. Sci. USA 81 :439-443; Herrin Jr., G.L and G.N. Bennett [1984] Gene 32 :349-356; Deuschle, U., et al. [1986] Proc. Natl. Acad. Sci. USA 83 :4134-4137; Hu, M.C-T. and N. Davidson [1988] Gene 62 :301-314; Figge, J., et al. [1988] Cell 52 :713-722). The subject invention is designed to improve lactose induction from such lac operated promoters.

This invention enables cells to make enough lacZ and Y protein to efficiently take up lactose and convert it to glucose and galactose, as well as to the true lac inducer, allolactose. In addition, the invention enables the cell to synthesize enough repressor (lacI protein) to bind to the lac operator upstream from a heterologous gene, and keep heterologous gene expression off in the absence of inducer. With excess lactose present in the medium, the cell can accumulate sufficient concentrations of allolactose for efficient derepression of the lac operator.

Brief Description of the Drawings

Figure 1 - pMYC 2005, LacI and Z operon fusion
A synthetic fragment of DNA was cloned into pUC18 to replace the normal sequence found at Hin d III-403 to Hae II-524. This synthetic sequence removes the lac promoter and operator sequences and replaces them with a Shine-Dalgarno site.
Figure 2 - pMYC 2101, construction of the lacIZYA operon
Three fragments of DNA were ligated and then introduced by transformation into MC1061 (Casadaban, M. and S. Cohen [1980] J. Mol. Biol. 138 :179-207) to construct the plasmid pMYC2101. The first fragment came from pMYC2005 as a Hin dIII-403 to Eae I-485 piece, the second fragment came from pMC9 as an Eco RI-1 to Eae I-1103 piece and the final fragment comes from pSKS107 as a 9889 bp Hin dIII-31 to Eco RI-1 piece. Once constructed, the Eco RI and Sal I ends were converted to Bam HI and Bgl II ends, respectively, using oligonucleotide linkers, creating pMYC2101-B.
Figure 3 - pMYC467, a tac promoted toxin-containing plasmid
The tac promoted toxin gene found in pMYC436 (NRRL deposit no. B-18292) was cloned as a 4.5 Kbp Bam HI to Pst I fragment in the vector pTJS260. Not all the sequence of pTJS260 or of the 3' flanking sequences of the toxin genes are available, hence some restriction sites are approximated in Figures 3, 4, 5 and 6.
Figure 4 - pMYC471, a lactose inducible plasmid
The lacIZYA operon was cloned into the Bam HI site of pMYC467. The resulting plasmid, pMYC471, was then induced by transformation into MB101, a P. fluorescens .
Figure 5 - pMYC485, an alternate lactose inducible plasmid
A lacI ^{Q}ZYA operon was constructed by replacing lacI with lacI ^{Q} as a 638 bp Bam HI to Apa I fragment. The lacI ^{Q}ZYA operon was then cloned into the Bam HI site of pMYC467. The resulting plasmid, pMYC485, was tested in MB101 for lactose inducibility. The lacI ^{Q}ZYA operon is shown in Table 1 wherein base 16 is a T instead of a C as shown in the table.
Figure 6 - pMYC1611, a coleopteran toxin-lactose inducible plasmid

The plasmid pMYC471 was cut with Bam HI (at position 12103) and Kpn I (at position 7933) to remove the tac -promoted lepidopteran-active toxin and then both ends were filled in with T4 DNA polymerase, resulting in blunt termini. The tac promoted toxin gene disclosed in U.S. Patent 4,771,131, was inserted into the above DNA as a blunt-ended Sca I (of pBR322) to filled-in Hin dIII fragment (at the 3' end of the coleopteran-active sequence). The 5' end of the tac promoter had been previously inserted by blunt-end ligation into the Eco RI site of pBR322.

Detailed Disclosure of the Invention

Upon removal of the CAP binding site and lac promoter/operator from the lac operon, there is realized an improved control of expression of a heterologous gene. Exemplified herein is the insertion of a lacIZYA fusion construct into a plasmid containing a tac -promoted gene encoding a lepidopteran insect toxin. The plasmid (pMYC47I) was then used to transform Pseudomonas fluorescens , which was designated Pseudomonas fluorescens MR471 (pMYC471).

One element of plasmid pMYC471 provides its host with constitutive levels of lac operon gene products. For this purpose, the operon could be fused to any of a variety of available constitutive promoters. In this instance, it was fused to the lacI promoter. In addition, a new construct containing a lacI ^{Q} promoter, instead of the lacI promoter, was made (pMYC485). This new construct provides higher levels of the repressor as well as of lac operon gene products. The plasmid, pMYC4BS, functions like pMYC471.

As a demonstration that lactose inducibility is not dependent on the specific gene under tac promoter control, another gene encoding a coleopteran-active toxin was cloned into the pMYC471 vector replacing the lepidopteran-active toxin gene, resulting in pMYC1161. Pseudomonas fluorescens strain MB101 carrying this new plasmid also permits lactose induction of toxin expression. This will hold true for any gene under control of a lac operated promoter.

Another aspect of this fusion is that the lac operon is no longer regulated by the normal control elements associated with lactose. This means that synthesis of lac operon ZYA gene products in these constructs (pMYC471, pMYC485, and pMYC1161) is constitutive and occurs independently of the concentrations of lactose or glucose present in the cell. For example, in the presence or absence of IPTG or lactose, a new protein band corresponding to the mass of β-galactosidase is apparent on SDS-PAGE and the clones cleave X-gal, forming blue colonies under the same conditions.

For ease in construction, the lacA gene has been included on all the plasmids contining lacIZYA mentioned in this disclosure. The lacA gene product has no role (Freifelder, supra) in production of allolactose and can easily be removed from the lacIZYA operon. For example, there is a non- unique Afl II (CTTAAG) restriction enzyme site a the stop codon of the lacY gene. By using this AFI II restriction site, it is possible to remove the lacA gene. The lacIZY operon has the same lactose induction properties as those found in the lacIZYA operon.

The data of Table 2 demonstrate toxin production is regulated by lactose or IPTG. Although the amount of lactose used for induction is 10 to 20 fold higher than IPTG induction levels, the significantly reduced cost of lactose ($7.00 per kilo, Sigma Chemical Co.) compared to IPTG ($20,000 per kilo, Sigma Chemical Co.) make the former inducer highly economically advantageous. For maximum production levels the metabolized substrate, lactose, must be replenished during cell growth or the culture will decrease the synthesis of the tac -promoted gene.

Materials and Methods

Cloning and DNA manipulation techniques are described in Maniatis, T., E.F. Fritsch and J. Sambrook (1982) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Publishers, Cold Spring Harbor, New York.

A plasmid containing the lac operon, pSKS107, was received from Dr. M. Casadaban at the University of Chicago (Shapira, S.K, J. Chou, F.V. Richaud and M.J. Casadaban [1983] Gene 25 :71-82). The sequence of the lac operon is published (Kalnins, A., K Otto, U. Ruther and B. Muller-Hill [1983] EMBO J. 2 :593-597; Hediger, M.A, D.F. Johnson, D.P. Nierlich and I. Zabin [1985] Pro. Nat. Acad. Sci. 82 :6414-6418). The lacI gene, on a 1.7KB Eco RI restriction fragment cloned into pBR322, is available from the ATCC, as pMC9, catalogue no. 37195, 37196 and 37197. The sequence of the lacI gene is also published (Farabaugh, P.J. [1978] Nature 274 :765-769). The broad host range vector, pMMB22, was received from Dr. M.

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Bagdasarian (Bagdasarian, M.M., E. Amann, R. Lurz, B. Ruckert and M Bagdasarian [1983] Gene 26 :273-282). A Hin dIII fragment of pMMB22, containing the lacI ^{Q} gene, was relinkered to Bam HI. The sequence of the lacI ^{Q} gene has also been published (Calos, M.P. [1978] Nature 274 :762-765). Analysis here is largely based on the published DNA sequences. Some of the critical portions of the constructs were confirmed by DNA sequencing, e.g., the lacI and lacI ^{Q} promoters and the fusion region between the lacI transcriptional unit and the lac operon. The vector, pUC18, is available from a variety of sources, such as catalogue no. 27-4949-01 of Pharmacia Corporation. The broad host range vector, pTJS260, is available from Dr. D.R. Helinski at the University of California, San Diego, La Jolla CA 92093 (Schmidhauser, T.J. [1986] Ph.D Thesis, UCSD).

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Fusions of the LacI Gene and Lac Operon.

Fusions of the lacI gene and lac operon were first carried out in pUC18. To do so, pUC18 was linearized with Hin dIII and then partially cut with Hae II. The DNA was resolved by electrophoresis on a 1.4% agarose gel. The 2402 bp band was eluted and ligated to a double-stranded synthetic DNA insert produced by β-cyanoester chemistry on an Applied Biosystems 380A oligonucleotide synthesizer. This sequence starts at BP 1073 of the lacI sequence (Farabaugh, P.J., supra) as seen in Table 1.

Hae II   Eae I

CC AAT ACG CAA ACC GCC TCT CCC CGC GCG TTG GCC GAT
CGCGGG TTA TGC GTT TGG CGG AGA GGG GCG CGC AAC CGG CTA

Xho I

TCA TTA ATG CAA CTC GCA CGA CAG GTC TCG AGA CTG GAA AGC
AGT AAT TAC GTT GAG CGT GCT GTC CAG AGC TCT GAC CTT TCG

lacI   lacZ
Stop   S/D   Start   Hind III

GGG CAG TGA GCGCTAGGAGGTAACTT ATG GAA
CCC GTC ACT CGCGATCCTCCATTGAA TAC CTTTCGA

The above synthetic sequence removes the Pvu II site normally found at BP 1123 of lacI . A new XhoI site was inserted near the 3' end of the lacI gene (BP 1137). These changes were introduced for ease in identification of the new construct. No amino acid changes would occur as a result of the mutations introduced in making the base pair changes for the above two restriction sites. In this construct, the distance between the stop codon of lacI and the start codon of lacZ is 17 base pairs. This region contains a ribosome binding site (marked as S/D) such that a ribosome translating the lacI transcript will be able to continue synthesis of the lacZ gene product from the same transcript. A plasmid diagram of pMYC2005 is seen in Figure 1.

Example 2 - Confirmation of Sequence.

The synthetic portion of pMYC2005 was sequenced to validate its structure and was used in a three-piece ligation to tie the full lacI gene of pMC9 to the lac operon found in pSKS107 (Figure 2). After transformation, the lacI/Z fusion region was sequenced from a blue colony on an LB + X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase, a colorimeteric substrate for the presence of β-galactosidase) plate. Those restriction sites that were examined yielded fragments of the predicted sized (Figure 2). The lacI

promoter region was also confirmed by sequence analysis.

Example 3 - Cloning.

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The Eco RI and Sal I ends of the lacIZYA construct (Figure 2) were respectively relinkered to yield Bam HI and Bgl II restriction sites. The lacIZYA operon was cloned into the unique Bam HI site of pMYC467 (Figure 3) yielding pMYC471 (Figure 4). The plasmid, pMYC467, contains the tac promoted lepidopteran toxin gene of pMYC436 (a cry IA(c)-like toxin gene [NRRL deposit no. B-18292] Adang, M.J. et al. [1985]

10 Gene 36 :289-300) cloned into the broad host range vector, pTJS260. pMYC471 was introduced by transformation into P. flourescens MB101 and the resulting clone was designated MR471. MR471 was tested for the key elements of the resident pMYC471 plasmid. A functional repressor is synthesized because in the absence of IPTG or lactose no significant amount of toxin is produced (Table 1). The cells are blue when plated on X-gal because a functional β-galactosidase is present. A functional permease and

15 β-galactosidase are present because the cells are able to induce the tac promoter in the presence of lactose (Table 1).

The subject invention can be used with any heterologous gene to control its expression from lac operated promoters. The expression product (protein) can be isolated from the culture medium of the producing microbe by means known in the art for isolating such a product from microbial cultures.

20 Alternatively, a product which remains intracellular can be used in the form of the microbe itself, for example, as a biological insecticide. See U.S. patents 4,695,455 and 4,695,462 for such uses.

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Table 1

<u>T</u> in lac I^Q

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   1  GACACCATCG AATGGCGCAA AACCTTTCGC GGTATGGCAT GATAGCGCCC
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start codon
lac I

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  51  GGAAGAGAGT CAATTCAGGG TGGTGAATGT GAAACCAGTA ACGTTATACG
 101  ATGTCGCAGA GTATGCCGGT GTCTCTTATC AGACCGTTTC CCGCGTGGTG
 151  AACCAGGCCA GCCACGTTTC TGCGAAAACG CGGGAAAAAG TGGAAGCGGC
 201  GATGGCGGAG CTGAATTACA TTCCCAACCG CGTGGCACAA CAACTGGCGG
 251  GCAAACAGTC GTTGCTGATT GGCGTTGCCA CCTCCAGTCT GGCCCTGCAC
 301  GCGCCGTCGC AAATTGTCGC GGCGATTAAA TCTCGCGCCG ATCAACTGGG
 351  TGCCAGCGTG GTGGTGTCGA TGGTAGAACG AAGCGGCGTC GAAGCCTGTA
 401  AAGCGGCGGT GCACAATCTT CTCGCGCAAC GCGTCAGTGG GCTGATCATT
 451  AACTATCCGC TGGATGACCA GGATGCCATT GCTGTGGAAG CTGCCTGCAC
 501  TAATGTTCCG GCGTTATTTC TTGATGTCTC TGACCAGACA CCCATCAACA
 551  GTATTATTTT CTCCCATGAA GACGGTACGC GACTGGGCGT GGAGCATCTG
 601  GTCGCATTGG GTCACCAGCA AATCGCGCTG TTAGCGGGCC CATTAAGTTC
 651  TGTCTCGGCG CGTCTGCGTC TGGCTGGCTG GCATAAATAT CTCACTCGCA
 701  ATCAAATTCA GCCGATAGCG GAACGGGAAG GCGACTGGAG TGCCATGTCC
 751  GGTTTTCAAC AAACCATGCA AATGCTGAAT GAGGGCATCG TTCCCACTGC
 801  GATGCTGGTT GCCAACGATC AGATGGCGCT GGGCGCAATG CGCGCCATTA
 851  CCGAGTCCGG GCTGCGCGTT GGTGCGGATA TCTCGGTAGT GGGATACGAC
 901  GATACCGAAG ACAGCTCATG TTATATCCCG CCGTCAACCA CCATCAAACA
 951  GGATTTTCGC CTGCTGGGGC AAACCAGCGT GGACCGCTTG CTGCAACTCT
1001  CTCAGGGCCA GGCGGTGAAG GGCAATCAGC TGTTGCCCGT CTCACTGGTG
1051  AAAAGAAAAA CCACCCTGGC GC'CCAATACG CAAACCGCCT CTCCCCGCGC
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Table 1 continued

1101 GTTGGCCGAT TCATTAATGC AACTCGCACG ACAGGTCTCG AGACTGGAAA

                 stop codon   start codon
                   lac I   lac Z   (1)
1151 GCGGGCAGTG AGCGCTAGGA GGTAACTTAT GGAA'AGCTTG GCACTGGCCG

1201 TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT

1251 CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC

1301 CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT GGCGAATGGC

1351 GCTTTGCCTG GTTTCCGGCA CCAGAAGCGG TGCCGGAAAG CTGGCTGGAG

1401 TGCGATCTTC CTGAGGCCGA TACTGTCGTC GTCCCCTCAA ACTGGCAGAT

1451 GCACGGTTAC GATGCGCCCA TCTACACCAA CGTAACCTAT CCCATTACGG

1501 TCAATCCGCC GTTTGTTCCC ACGGAGAATC CGACGGGTTG TTACTCGCTC

1551 ACATTTAATG TTGATGAAAG CTGGCTACAG GAAGGCCAGA CGCGAATTAT

1601 TTTTGATGGC GTTAACTCGG CGTTTCATCT GTGGTGCAAC GGGCGCTGGG

1651 TCGGTTACGG CCAGGACAGT CGTTTGCCGT CTGAATTTGA CCTGAGCGCA

1701 TTTTTACGCG CCGGAGAAAA CCGCCTCGCG GTGATGGTGC TGCGTTGGAG

1751 TGACGGCAGT TATCTGGAAG ATCAGGATAT GTGGCGGATG AGCGGCATTT

1801 TCCGTGACGT CTCGTTGCTG CATAAACCGA CTACACAAAT CAGCGATTTC

1851 CATGTTGCCA CTCGCTTTAA TGATGATTTC AGCCGCGCTG TACTGGAGGC

1901 TGAAGTTCAG ATGTGCGGCG AGTTGCGTGA CTACCTACGG GTAACAGTTT

1951 CTTTATGGCA GGGTGAAACG CAGGTCGCCA GCGGCACCGC GCCTTTCGGC

2001 GGTGAAATTA TCGATGAGCG TGGTGGTTAT GCCGATCGCG TCACACTACG

2051 TCTGAACGTC GAAAACCCGA AACTGTGGAG CGCCGAAATC CCGAATCTCT

2101 ATCGTGCGGT GGTTGAACTG CACACCGCCG ACGGCACGCT GATTGAAGCA

2151 GAAGCCTGCG ATGTCGGTTT CCGCGAGGTG CGGATTGAAA ATGGTCTGCT

2201 GCTGCTGAAC GGCAAGCCGT TGCTGATTCG AGGCGTTAAC CGTCACGAGC

2251 ATCATCCTCT GCATGGTCAG GTCATGGATG AGCAGACGAT GGTGCAGGAT

2301 ATCCTGCTGA TGAAGCAGAA CAACTTTAAC GCCGTGCGCT GTTCGCATTA

2351 TCCGAACCAT CCGCTGTGGT ACACGCTGTG CGACCGCTAC GGCCTGTATG

Table 1 continued

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2401   TGGTGGATGA AGCCAATATT GAAACCCACG GCATGGTGCC AATGAATCGT
2451   CTGACCGATG ATCCGCGCTG GCTACCGGCG ATGAGCGAAC GCGTAACGCG
2501   AATGGTGCAG CGCGATCGTA ATCACCCGAG TGTGATCATC TGGTCGCTGG
2551   GGAATGAATC AGGCCACGGC GCTAATCACG ACGCGCTGTA TCGCTGGATC
2601   AAATCTGTCG ATCCTTCCCG CCCGGTGCAG TATGAAGGCG GCGGAGCCGA
2651   CACCACGGCC ACCGATATTA TTTGCCCGAT GTACGCGCGC GTGGATGAAG
2701   ACCAGCCCTT CCCGGCTGTG CCGAAATGGT CCATCAAAAA ATGGCTTTCG
2751   CTACCTGGAG AGACGCGCCC GCTGATCCTT TGCGAATACG CCCACGCGAT
2801   GGGTAACAGT CTTGGCGGTT TCGCTAAATA CTGGCAGGCG TTTCGTCAGT
2851   ATCCCCGTTT ACAGGGCGGC TTCGTCTGGG ACTGGGTGGA TCAGTCGCTG
2901   ATTAAATATG ATGAAAACGG CAACCCGTGG TCGGCTTACG GCGGTGATTT
2951   TGGCGATACG CCGAACGATC GCCAGTTCTG TATGAACGGT CTGGTCTTTG
3001   CCGACCGCAC GCCGCATCCA GCGCTGACGG AAGCAAAACA CCAGCAGCAG
3051   TTTTTCCAGT TCCGTTTATC CGGGCAAACC ATCGAAGTGA CCAGCGAATA
3101   CCTGTTCCGT CATAGCGATA ACGAGCTCCT GCACTGGATG GTGGCGCTGG
3151   ATGGTAAGCC GCTGGCAAGC GGTGAAGTGC CTCTGGATGT CGCTCCACAA
3201   GGTAAACAGT TGATTGAACT GCCTGAACTA CCGCAGCCGG AGAGCGCCGG
3251   GCAACTCTGG CTCACAGTAC GCGTAGTGCA ACCGAACGCG ACCGCATGGT
3301   CAGAAGCCGG GCACATCAGC GCCTGGCAGC AGTGGCGTCT GGCGGAAAAC
3351   CTCAGTGTGA CGCTCCCCGC CGCGTCCCAC GCCATCCCGC ATCTGACCAC
3401   CAGCGAAATG GATTTTTGCA TCGAGCTGGG TAATAAGCGT TGGCAATTTA
3451   ACCGCCAGTC AGGCTTTCTT TCACAGATGT GGATTGGCGA TAAAAAACAA
3501   CTGCTGACGC CGCTGCGCGA TCAGTTCACC CGTGCACCGC TGGATAACGA
3551   CATTGGCGTA AGTGAAGCGA CCCGCATTGA CCCTAACGCC TGGGTCGAAC
3601   GCTGGAAGGC GGCGGGCCAT TACCAGGCCG AAGCAGCGTT GTTGCAGTGC
3651   ACGGCAGATA CACTTGCTGA TGCGGTGCTG ATTACGACCG CTCACGCGTG
3701   GCAGCATCAG GGGAAAACCT TATTTATCAG CCGGAAAACC TACCGGATTG
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Table 1 continued

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3751   ATGGTAGTGG  TCAAATGGCG  ATTACCGTTG  ATGTTGAAGT  GGCGAGCGAT

3801   ACACCGCATC  CGGCGCGGAT  TGGCCTGAAC  TGCCAGCTGG  CGCAGGTAGC

3851   AGAGCGGGTA  AACTGGCTCG  GATTAGGGCC  GCAAGAAAAC  TATCCCGACC

3901   GCCTTACTGC  CGCCTGTTTT  GACCGCTGGG  ATCTGCCATT  GTCAGACATG

3951   TATACCCCGT  ACGTCTTCCC  GAGCGAAAAC  GGTCTGCGCT  GCGGGACGCG

4001   CGAATTGAAT  TATGGCCCAC  ACCAGTGGCG  CGGCGACTTC  CAGTTCAACA

4051   TCAGCCGCTA  CAGTCAACAG  CAACTGATGG  AAACCAGCCA  TCGCCATCTG

4101   CTGCACGCGG  AAGAAGGCAC  ATGGCTGAAT  ATCGACGGTT  TCCATATGGG
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mutated EcoRI
site

```
4151   GATTGGTGGC  GACGACTCCT  GGAGCCCGTC  AGTATCGGCG  NNNNNNCAGC
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stop codon
lac Z

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4201   TGAGCGCCGG  TCGCTACCAT  TACCAGTTGG  TCTGGTGTCA  AAAATAATAA
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start codon
lac Y

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4251   TAACCGGGCA  GGCCATGTCT  GCCCGTATTT  CGCGTAAGGA  AATCCATTAT

4301   GTACTATTTA  AAAAACACAA  ACTTTTGGAT  GTTCGGTTTA  TTCTTTTTCT

4351   TTTACTTTTT  TATCATGGGA  GCCTACTTCC  CGTTTTTCCC  GATTTGGCTA

4401   CATGACATCA  ACCATATCAG  CAAAAGTGAT  ACGGGTATTA  TTTTTGCCGC

4451   TATTTCTCTG  TTCTCGCTAT  TATTCCAACC  GCTGTTTGGT  CTGCTTTCTG

4501   ACAAACTCGG  GCTGCGCAAA  TACCTGCTGT  GGATTATTAC  CGGCATGTTA

4551   GTGATGTTTG  CGCCGTTCTT  TATTTTTATC  TTCGGGCCAC  TGTTACAATA

4601   CAACATTTTA  GTAGGATCGA  TTGTTGGTGG  TATTTATCTA  GGCTTTTGTT

4651   TTAACGCCGG  TGCGCCAGCA  GTAGAGGCAT  TTATTGAGAA  AGTCAGCCGT

4701   CGCAGTAATT  TCGAATTTGG  TCGCGCGCGG  ATGTTTGGCT  GTGTTGGCTG

4751   GGCGCTGTGT  GCCTCGATTG  TCGGCATCAT  GTTCACCATC  AATAATCAGT

4801   TTGTTTTCTG  GCTGGGCTCT  GGCTGTGCAC  TCATCCTCGC  CGTTTTACTC

4851   TTTTTCGCCA  AAACGGATGC  GCCCTCTTCT  GCCACGGTTG  CCAATGCGGT

4901   AGGTGCCAAC  CATTCGGCAT  TTAGCCTTAA  GCTGGCACTG  GAACTGTTCA
```

Table 1 continued

```
4951  GACAGCCAAA  ACTGTGGTTT  TTGTCACTGT  ATGTTATTGG  CGTTTCCTGC

5001  ACCTACGATG  TTTTTGACCA  ACAGTTTGCT  AATTTCTTTA  CTTCGTTCTT

5051  TGCTACCGGT  GAACAGGGTA  CGCGGGTATT  TGGCTACGTA  ACGACAATGG

5101  GCGAATTACT  TAACGCCTCG  ATTATGTTCT  TTGCGCCACT  GATCATTAAT

5151  CGCATCGGTG  GGAAAAACGC  CCTGCTGCTG  GCTGGCACTA  TTATGTCTGT

5201  ACGTATTATT  GGCTCATCGT  TCGCCACCTC  AGCGCTGGAA  GTGGTTATTC

5251  TGAAAACGCT  GCATATGTTT  GAAGTACCGT  TCCTGCTGGT  GGGCTGCTTT

5301  AAATATATTA  CCAGCCAGTT  TGAAGTGCGT  TTTTCAGCGA  CGATTTATCT

5351  GGTCTGTTTC  TGCTTCTTTA  AGCAACTGGC  GATGATTTTT  ATGTCTGTAC

5401  TGGCGGGCAA  TATGTATGAA  AGCATCGGTT  CCAGGGCGC  TTATCTGGTG

5451  CTGGGTCTGG  TGGCGCTGGG  CTTCACCTTA  ATTTCCGTGT  TCACGCTTAG
```

                                                    stop codon
                                                    lac Y

```
5501  CGGCCCCGGC  CCGCTTTCCC  TGCTGCGTCG  TCAGGTGAAT  GAAGTCGCTT

5551  AAGCAATCAA  TGTCGGATGC  GGCGCGACGC  TTATCCGACC  AACATATCAT
```

                        start codon
                        lac A

```
5601  AACGGAGTGA  TCGCATTGAA  CATGCCAATG  ACCGAAAGAA  TAAGAGCAGG

5651  CAAGCTATTT  ACCGATATGT  GCGAAGGCTT  ACCGGAAAAA  AGACTTCGTG

5701  GGAAAACGTT  AATGTATGAG  TTTAATCACT  CGCATCCATC  AGAAGTTGAA

5751  AAAAGAGAAA  GCCTGATTAA  AGAAATGTTT  GCCACGGTAG  GGGAAAACGC

5801  CTGGGTAGAA  CCGCCTGTCT  ATTTCTCTTA  CGGTTCCAAC  ATCCATATAG

5851  GCCGCAATTT  TTATGCAAAT  TTCAATTTAA  CCATTGTCGA  TGACTACACG

5901  GTAACAATCG  GTGATAACGT  ACTGATTGCA  CCCAACGTTA  CTCTTTCCGT

5951  TACGGGACAC  CCTGTACACC  ATGAATTGAG  AAAAAACGGC  GAGATGTACT

6001  CTTTTCCGAT  AACGATTGGC  AATAACGTCT  GGATCGGAAG  TCATGTGGTT

6051  ATTAATCCAG  GCGTCACCAT  CGGGGATAAT  TCTGTTATTG  GCGCGGGTAG

6101  TATCGTCACA  AAAGACATTC  CACCAAACGT  CGTGGCGGCT  GGCGTTCCTT

6151  GTCGGGTTAT  TCGCGAAATA  AACGACCGGG  ATAAGCACTA  TTATTTCAAA
```

Table 1 continued

```
                                         stop codon
                                            lac A
      6201   GATTATAAAG TTGAATCGTC AGTTTAAATT ATAAAAATTG CCTGATACGC

      6251   TGCGCTTATC AGGCCTACAA GTTCAGCGAT CTACATTAGC CGCATCCGGC

      6301   ATGAACAAAG CGCAGGAACA AGCGTCGCAT CATGCCTCTT TGACCCACAG

      6351   CTGCGGAAAA CGTACTGGTG CAAAACGCAG GGTTATGATC ATCAGCCCAA

      6401   CGACGCACAG CGCATGAAAT GCCCAGTCCA TCAGGTAATT GCCGCTGATA

      6451   CTACGCAGCA CGCCAGAAAA CCACGGGGCA AGCCCGGCGA TGATAAAACC

      6501   GATTCCCTGC ATAAACGCCA CCAGCTTGCC AGCAATAGCC GGTTGCACAG

      6551   AGTGATCGAG CGCCAGCAGC AAACAGAGCG GAAACGCGCC GCCCAGACCT

      6601   AACCCACACA CCATCGCCCA CAATACCGGC AATTGCATCG GCAGCCAGAT

      6651   AAAGCCGCAG AACCCCACCA GTTGTAACAC CAGCGCCAGC ATTAACAGTT

      6701   TGCGCCGATC CTGATGGCGA GCCATAGCAG GCATCAGCAA AGCTCCTGCG

      6751   GCTTGCCCAA GCGTCATCAA TGCCAGTAAG GAACCGCTGT ACTGCGCGCT

      6801   GGCACCAATC TCAATATAGA AAGCGGGTAA CCAGGCAATC AGGCTGGCGT

      6851   AACCGCCGTT AATCAGACCG AAGTAAACAC CCAGCGTCCA CGCGCGGGGA

      6901   GTGAATACCA CGCGAACCGG AGTGGTTGTT GTCTTGTGGG AAGAGGCGAC

      6951   CTCGCGGGCG CTTTGCCACC ACCAGGCAAA GAGCGCAACA ACGGCAGGCA

      7001   GCGCCACCAG GCGAGTGTTT GATACCAGGT TTCGCTATGT TGAACTAACC

      7051   AGGGCGTTAT GGCGGCACCA AGCCCACCGC CGCCCATCAG AGCCGCGGAC

      7101   CACAGCCCCA TCACCAGTGG CGTGCGCTGC TGAAACCGCC GTTTAATCAC

      7151   CGAAGCATCA CCGCCTGAAT GATGCCGATC CCCACCCCAC CAAGCAGTGC

      7201   GCTGCTAAGC AGCAGCGCAC TTTGCGGGTA AAGCTCACGC ATCAATGCAC

      7251   CGACGGCAAT CAGCAACAGA CTGATGGCGA CACTGCGACG TTCGCTGACA

      7301   TGCTGATGAA GCCAGCTTCC GGCCAGCGCC AGCCCGCCCA TGGTAACCAC

                      SalI site in pSKS107
      7351   CGGCAGAGCG GTCGAC
```

(1) sequence in bold, between the two ' marks, is synthetic DNA used to fuse the lac operon to the lac I or lac I^Q gene operon.

(2) pSKS107 contains an unsequenced mutation at the EcoRI site normally found in the lac Z gene.

Table 2.

Lactose Inducibility of Various Constructs			
Hours after[1] Induction	Lactose (mM)	Cells/ml	Toxin[2] (ug/ml)
MR471			
40	0	1.6×10^{10}	none detected
40 no lactose	2 mM IPTG	2.3×10^{10}	1003
15	20	9.1×10^{9}	1011
24	20	1.2×10^{10}	737
15	40	1.8×10^{10}	887
24	40	1.1×10^{10}	1025
23[3]	8.3[4]	1.9×10^{10}	938
39[3]	8.3[4]	2.0×10^{10}	1555
MB101 containing pMYC485			
24	40	-	775
24	0	-	none detected
MB101 containing pMYC1161			
24	40	-	300
24	0	-	none detected

(1) Cultures were induced upon reaching stationary phase.
(2) Toxin concentration was determined by laser densitometry of Coomassie-stained protein bands after electrophoresis of disrupted cells on SDS-PAGE (LKB Instructional Manual 2222-010).
(3) This experiment used MR471 grown in a 10L fermentor. All other experimental data were generated using same medium in 250 ml baffled shake flasks.
(4) In the fermentor, 8.3 mM lactose/hour was fed into the culture. It was found that MR471 did not metabolize this lactose level in a fermentor, resulting in increased concentrations during the experiment. The experiments done in hake flasks were given a single dose of lactose or IPTG at the indicated times.

Claims

1. A modified lac operon, wherein the CAP binding site for catabolite repression and the promoter/operator are deleted, and wherein the lac I gene is fused to the ZY or ZYA gene, which substantially retains the parent operon's ability to control expression of a heterologous gene.

2. An operon according to claim 1, having the sequence shown in Table 1, or a mutation thereof.

3. A DNA construct comprising an operon according to claim 1 or claim 2 and also the heterologous gene.

4. A transfer vector, e.g. a plasmid, comprising an operon or DNA as defined in any preceding claim.

5. A microbial host transformed by the transfer vector of claim 4.

6. A process for preparing a polypeptide encoded by the heterologous gene, which comprises culturing a microbe comprising DNA as defined in claim 3.
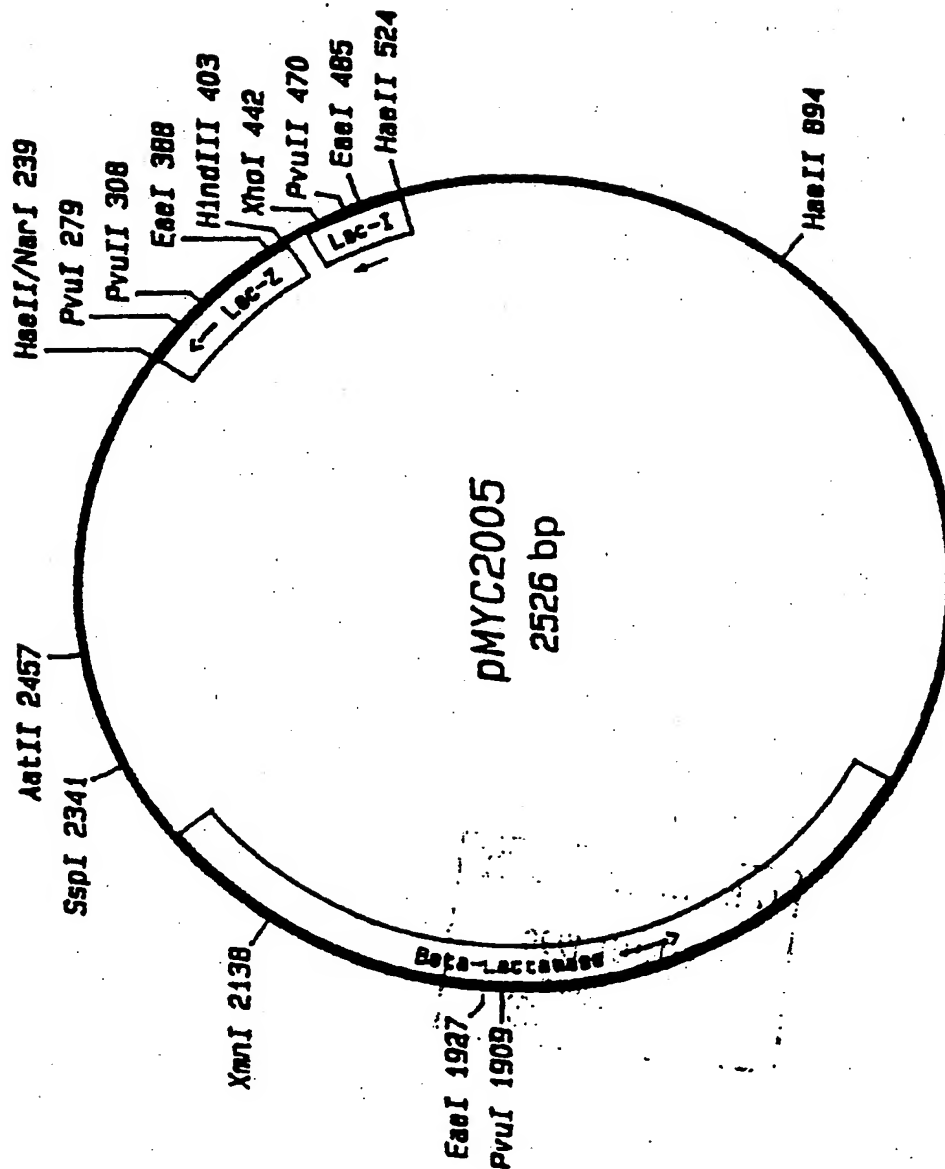
pMYC2005
2526 bp

HaeII/NarI 239
PvuI 279
PvuII 308
EaeI 388
HindIII 403
XhoI 442
PvuII 470
EaeI 485
HaeII 524

← Lac-z
Lac-i ←

HaeII 894

AatII 2457
SspI 2341
XmnI 2138
EaeI 1927
PvuI 1909

Beta-Lactamase

Figure 1
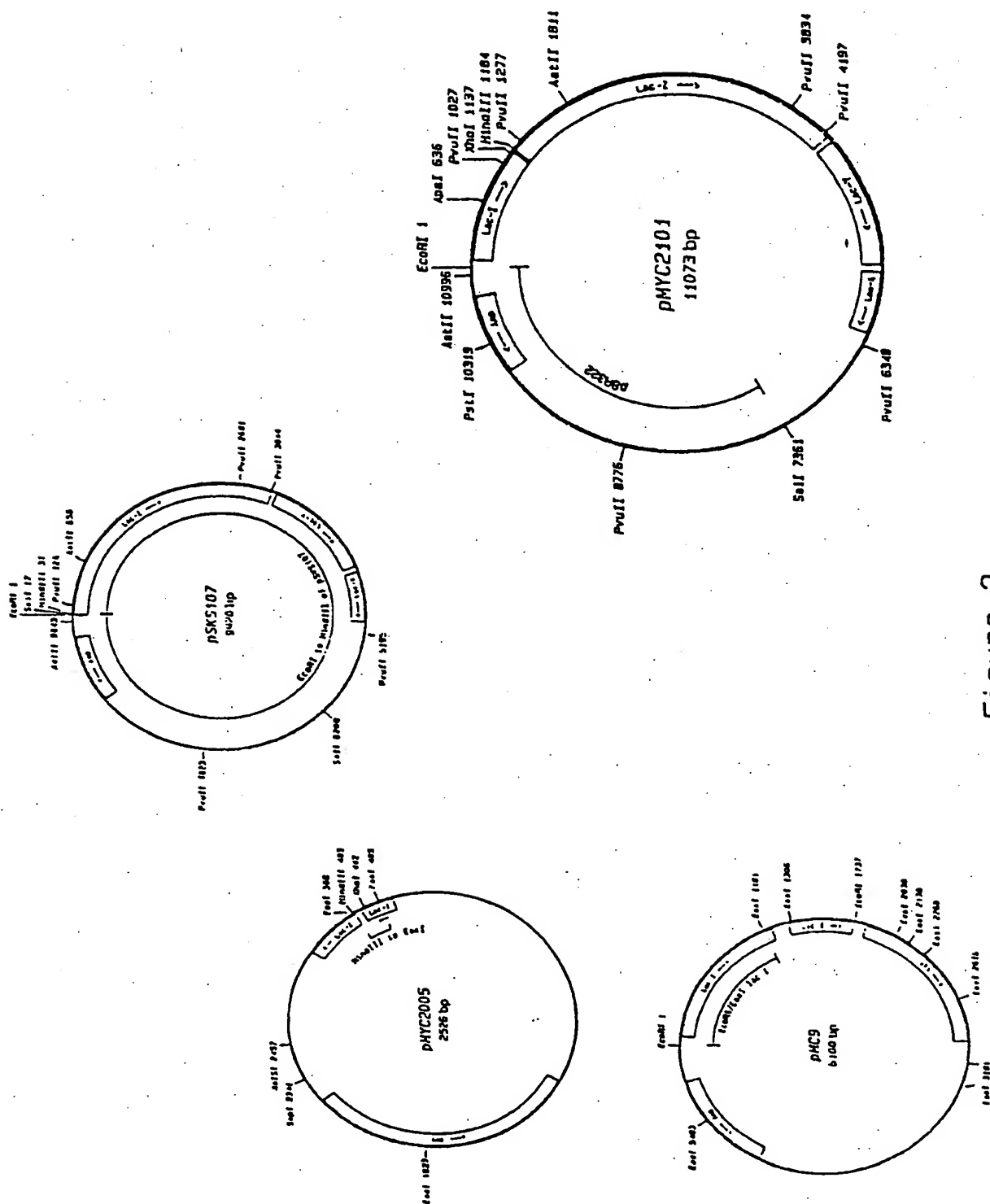
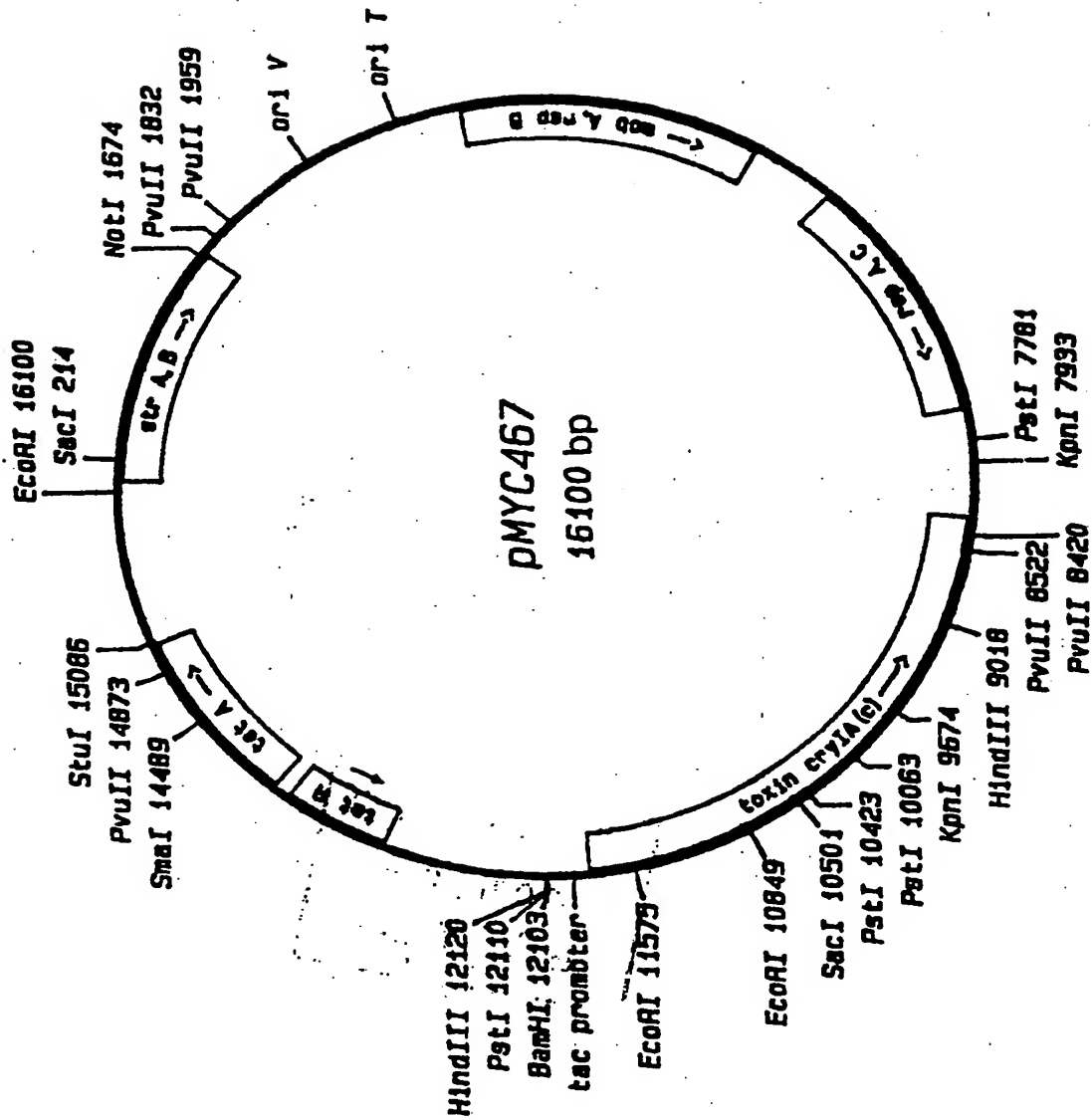PLASMIDMAP of: pMYC2005 check: 4484 from: 1 to: 2526

Figure 2

Figure 3

PLASMIDMAP of: pMYC467 check: 9500 from 1 to 16100

Figure 4

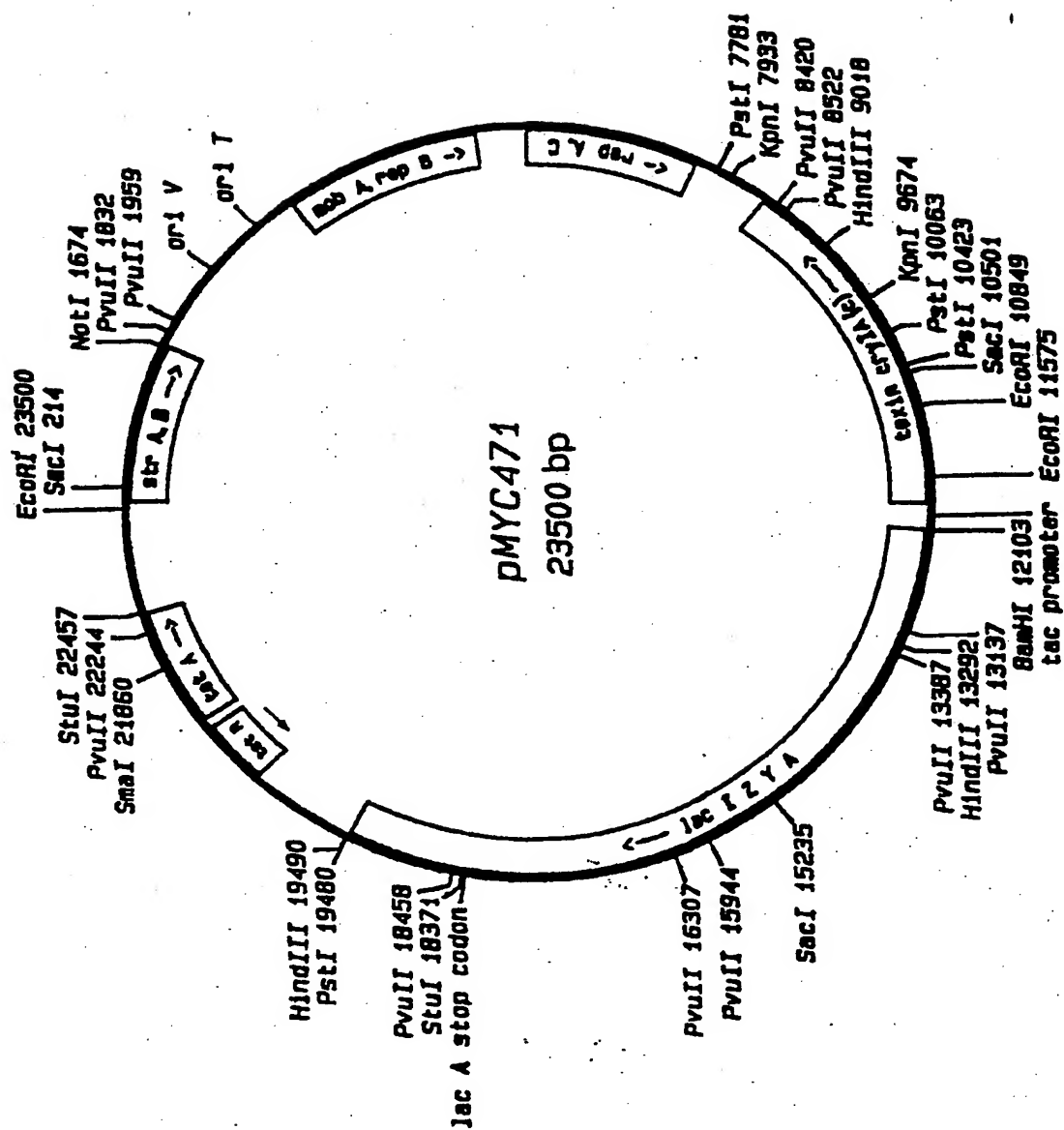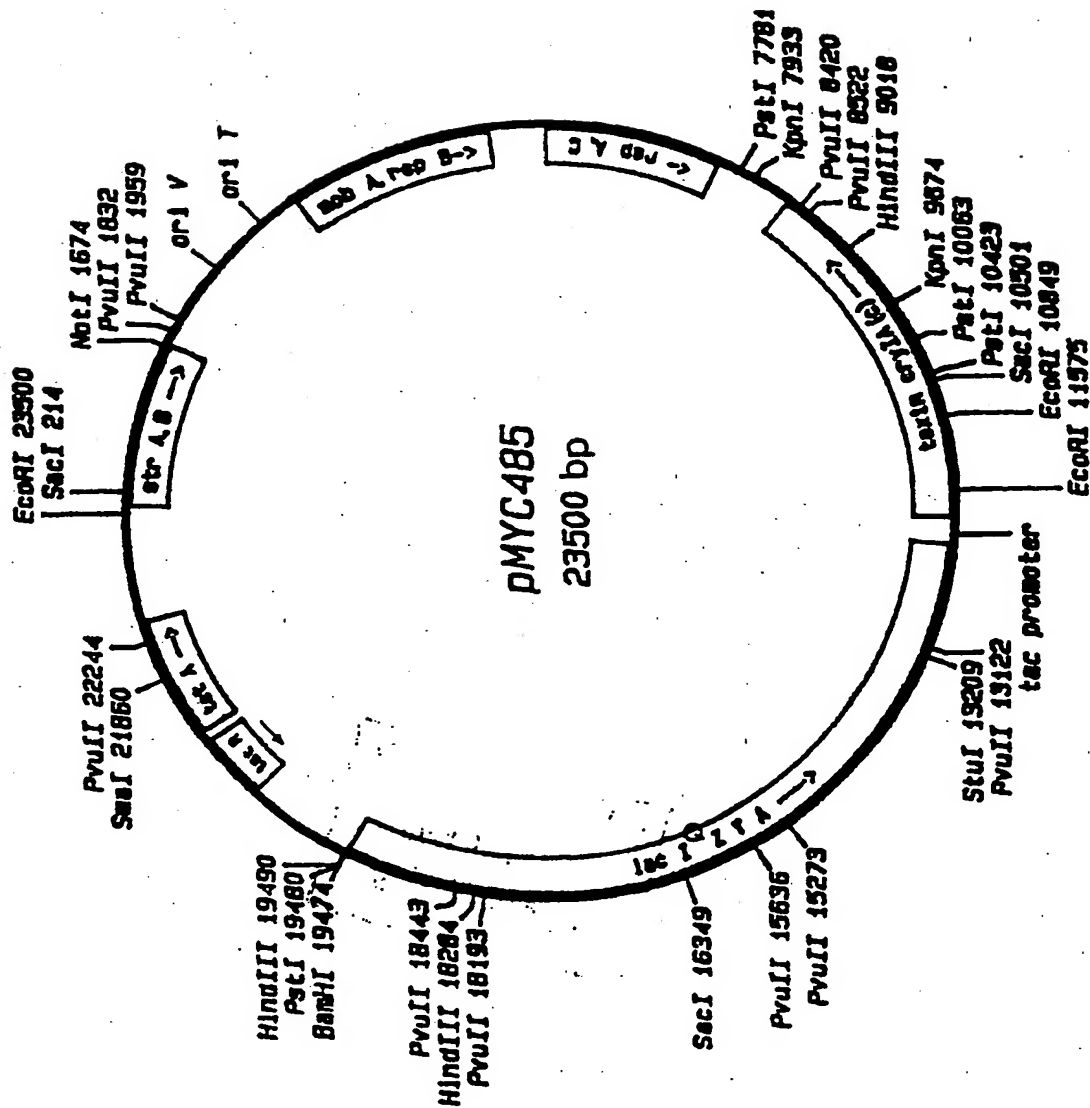PLASMIDMAP of: pMYC471 check: 3500 from 1 to: 23500

Figure 5

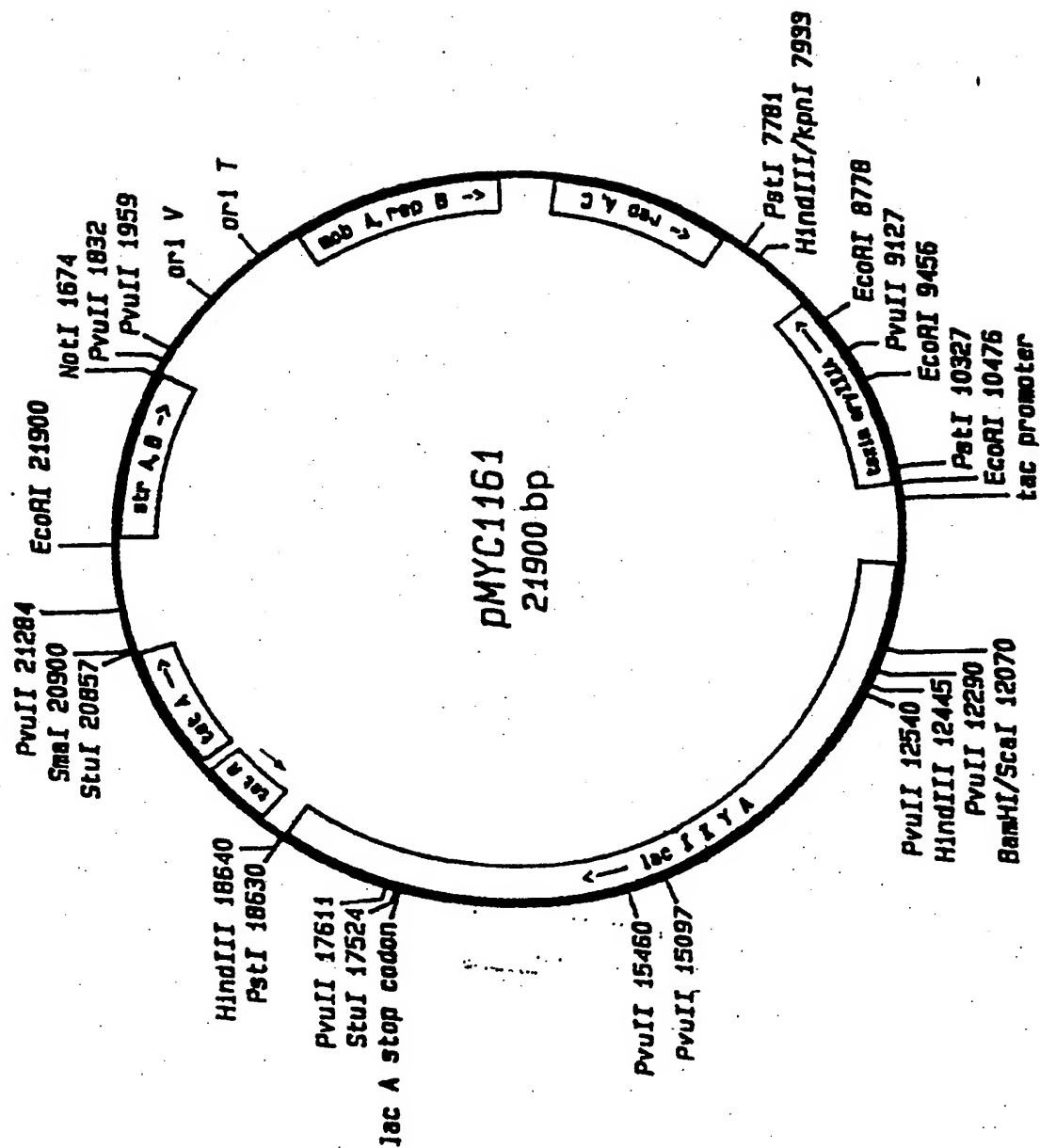PLASMIDMAP of: pMYC485 check: 3500 from: 1 to: 23500

pMYC1161
21900 bp

mob A rep B ->
<- rep A C

PstI 7781
HindIII/KpnI 7999
EcoRI 8778
PvuII 9127
EcoRI 9456
PstI 10327
EcoRI 10476
tac promoter

ori T
ori V
PvuII 1959
PvuII 1832
NotI 1674
EcoRI 21900
str A,B ->

PvuII 21284
SmaI 20900
StuI 20857
str A B

HindIII 18640
PstI 18630
PvuII 17611
StuI 17524
lac A stop codon
lac I I Y A
PvuII 15460
PvuII 15097

PvuII 12540
HindIII 12445
PvuII 12290
BamHI/ScaI 12070

Figure 6

PLASMIDMAP of: pMYC1161 check: 3500 from: 1 to: 21900

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European
Patent Office

**EUROPEAN SEARCH
REPORT**

Application Number

EP 90 30 7952

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	GENES & DEVELOPMENT, vol. 1, no. 3, 1987, pages 227-237, New York, US; J.K. WHORISKEY et al.: "Genetic rearrangements and gene amplification in Escherichia coli: DNA sequences at the junctures of amplified gene fusions" * Abstract; page 228, column 2, line 26 - page 229, column 2, line 24; page 229, figure 3 *	1,2	C 12 N 15/72 C 12 N 1/21
A	JOURNAL OF MOLECULAR BIOLOGY, vol. 186, no. 4, 1985, pages 733-742, London, GB; K.C. CONE et al.: "Functional analysis of lac repressor restart sites in translational initiation and reinitiation" * Abstract; page 735, column 1, line 40 - page 736, column 1, line 8; page 739, column 2, line 28 - page 740, column 1, line 13 *	1,2	
A	NUCLEIC ACIDS RESEARCH, vol. 12, no. 13, 1984, pages 5449-5464, Oxford, GB; X.-M. YU et al.: "Deletion analysis of the CAP-cAMP binding site of the Escherichia coli lactose promoter" * Abstract; page 84, line 1 - page 85, line 5 *	1	

TECHNICAL FIELDS
SEARCHED (Int. Cl.5)

C 12 N 15/00

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	17 October 90	GURDJIAN D P M

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